

EFFECTS OF MERCURY UPTAKE ON FLUCTUATING ASYMMETRY AND  
BEHAVIOR IN THE TERRESTRIAL ISOPOD *ARMADILLIDIUM VULGARE*

by

CAROL JEAN MYERS FLAUTE

(Under the Direction of Alan P. Covich)

ABSTRACT

Fluctuating asymmetry (FA) is an indicator of developmental instability. In this study, young terrestrial isopods (*Armadillidium vulgare* (Brandt 1833)) were exposed to food contaminated with HgCl<sub>2</sub>. After 12 weeks of exposure, FA was measured by comparing the number of compound eye ommatidia on the left and right sides of the head. In addition, to provide an ecologically relevant endpoint for comparison with FA data, four locomotory behavior traits (total distance traveled, proportion of time spent in motion, rate of travel while in motion, and proportion of time away from arena edge) were measured using video analysis of individual behavior trials. FA increased with increasing Hg concentration. While no statistically significant differences in behavioral traits were observed, total distance traveled appeared to increase with increasing Hg concentration.

INDEX WORDS: Ecotoxicology, terrestrial isopods, mercury, fluctuating asymmetry, locomotory behavior

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## DEDICATION

This thesis is dedicated to Keith, for having enough faith and support to move all the way to Georgia with me, and to my family for the value they have placed on academics throughout my life.

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## CHAPTER 1

### OVERVIEW OF THESIS STRUCTURE

This thesis describes an ecotoxicological study of the relationships between fluctuating asymmetry and behavior, using terrestrial isopods as model organisms and HgCl<sub>2</sub> as the contaminant of interest. Chapter 2 contains a focused review of the relevant literature pertaining to this study. Topics include: 1) the suitability of terrestrial isopods for ecotoxicological research; 2) HgCl<sub>2</sub>, including its distribution and abundance in soil, known effects on isopods and other organisms, and the possible role of isopods and HgCl<sub>2</sub> in the formation of methyl mercury; 3) fluctuating asymmetry, including previous studies involving isopods; and 4) support for studying behavior as an ecologically relevant component of this type of study.

Chapters 3-6 will serve as the foundation of a manuscript to be submitted to *Environmental Toxicology and Chemistry*, describing a study on the effects of Hg uptake on fluctuating asymmetry and locomotory behavior in the terrestrial isopod *Armadillidium vulgare* (Brandt, 1833). The manuscript will be entitled “Effects of mercury uptake on fluctuating asymmetry and behavior in terrestrial isopods.”

These chapters are followed by a literature cited section, as well as appendices that provide additional information on preliminary research leading to this study, additional results, and lessons learned that should be considered before undertaking future research building upon the information presented in this thesis.

## CHAPTER 2

### LITERATURE REVIEW

#### *The use of terrestrial isopods in soil ecotoxicology*

Terrestrial isopods provide a convenient model system for studying the effects of contaminants [1-4] because they are abundant, widespread, easily identified, and have direct contact with numerous soil pollutants [4]. Terrestrial isopods play important roles in decomposition processes such as mechanical and chemical breakdown of leaf litter and enhancement of microbial activity. They are primarily saprophagous, but are also coprophagous and occasionally predatory [5,6]. This varied diet allows for many possible routes of contaminant uptake. In addition, they are consumed by a variety of predators such as birds, frogs, lizards, shrews, spiders, ground beetles, scorpions, and chilopods [4]. Their varied diet and wide variety of predators, combined with the fact that many metals (including Hg) bioaccumulate in the hepatopancreas [7], suggest that they may play an important role in trophic transfer of contaminants.

Terrestrial isopods sequester Hg and other metals in the hepatopancreas, which isolates these potentially toxic metals from important biological molecules. Hg is stored in a type B granule, which is a high-density inclusion body originating from the lysosomal system and consisting primarily of acid phosphatase. Metal storage in the hepatopancreas is efficient, leaving low concentrations in body fluids. When the storage capacity of the hepatopancreas is exceeded, or when uptake rate exceeds the rate of storage, direct metal toxicity occurs [7]. Previous research with the terrestrial isopod

*Oniscus asellus* suggests a severe energy lack in the metabolic tissues of individuals from some metal contaminated sites, which evidence suggests is due to the high metabolic demands of metal sequestration in the hepatopancreas of this species. In contrast, metal storage in the hepatopancreas does not appear to be energy intensive in the terrestrial isopod *Porcelio scaber* [8]. In this study, I investigated developmental and behavioral effects of dietary Hg on the terrestrial isopod *Armadillidium vulgare*. Energy limitation may be an important factor in the process underlying fluctuating asymmetry, the indicator of developmental instability measured in the current study [9]. However, the energetics of this process does not appear to have been studied for *A. vulgare*.

### *Mercury*

Because soils have a high capacity to sequester Hg, they serve as a sink for atmospheric Hg. Clay soils and organic soil horizons have an especially high affinity for adsorption of atmospherically deposited Hg.  $\text{HgCl}_2$  is one of the most common forms of Hg found across all soil pH ranges [10]. Hg concentrates at relatively shallow depths in the soil [10] where terrestrial isopods are most abundant [4]. Thus, these isopods are well-suited for studying soil Hg contamination. However, few studies have investigated the effects of Hg on isopods. Both chronic exposure to Hg contamination in the field and acute laboratory exposure to  $\text{HgCl}_2$  were found to correlate with a change in gut bacteria community composition in the terrestrial isopod *Porcellio scaber* [11]. In the aquatic isopod *Asellus aquaticus*,  $\text{HgCl}_2$  inhibited unidirectional influx of Ca and Na [12]. In *in vitro* studies using a variety of other species, mercuric compounds have been shown to inhibit enzyme function affect the immune system, and block cellular receptors and ion

channels. By blocking these receptors and channels, mercuric compounds indirectly affect intracellular signaling processes that are important to the nervous system [13].

This study focuses on toxic effects of HgCl<sub>2</sub>. However, HgCl<sub>2</sub> is also important to study in soil systems due to the possibility of methylation. While methyl Hg (CH<sub>3</sub>Hg<sup>+</sup>) generally makes up less than 2% of total soil Hg, it is of great concern in ecotoxicology because it is extremely toxic, biomagnifies through food webs even at low concentrations, and inorganic Hg compounds such as HgCl<sub>2</sub> can be biotransformed to methyl Hg by soil bacteria [10]. Because terrestrial isopods are known to increase soil microbial activity [6], they may play an indirect role in this process. In addition, methylation has been found to occur within the gut of *P. scaber* at low levels; however, demethylation occurs at similar rates and so there does not appear to be a net increase in methyl Hg due to isopod gut flora [14,15].

### *Fluctuating asymmetry*

In this study, I was interested in the effects of chronic exposure to HgCl<sub>2</sub> via the diet on the symmetry and movement of *A. vulgare*. Fluctuating asymmetry (FA), defined as random deviations from bilateral symmetry, is used as an indicator of developmental instability due to environmental stress in biological organisms [16,17]. FA indices are useful measures of developmental instability for several reasons. First, because comparisons are made within a single individual, development of a pair of bilaterally symmetrical traits is controlled by a single gene complex, controlling for genetic heterogeneity. Second, since two bilaterally symmetrical traits grow at the same time, they grow under homogeneous environmental conditions. Third, prediction of the

optimal phenotype can be determined *a priori* because bilaterally symmetrical traits have an optimal phenotype that has zero asymmetry by definition. Thus, bilateral symmetry facilitates between-site comparisons by controlling for genetic heterogeneity and environmental variation and eliminating the need for preliminary studies to determine the optimal phenotype for each site [9]. Finally, FA is relatively easy and inexpensive to measure [18,19].

FA has long been assumed to be a predictor of fitness; however, there is considerable debate over whether this assumption consistently holds true [9,20-29]. Many possible explanations have been given for this discrepancy, such as interactions between fitness components, interactions between genotype and environment, and heterogeneity in the relationship between individual quality and fitness [29].

For terrestrial isopods, FA research has been limited to only two previous studies. Velisics et al. [30] measured six traits in *Trachelipus rathkii* (including ommatidia number) and found that all were consistent and suitable for use in FA analyses. E.L. Peters (University of Chicago, IL, USA, personal communication) examined FA in compound eye lenses (ommatidia) in wild populations of *A. vulgare* and found that asymmetry increased with increasing body burdens of several metals, including Hg. I sought to determine if results from the study by Peters could be induced in a laboratory setting by feeding HgCl<sub>2</sub> to young, developing isopods. If so, this effect would further support the suitability of FA in *A. vulgare* ommatidia as a bioindicator of Hg contamination.

## *Behavior*

Ecotoxicological studies are often criticized for a lack of ecological relevance, particularly in terms of linking effects at the individual and within-organism levels of organization with effects at population, community, and ecosystem levels [e.g. 31]. Behavioral studies are effective integrators of levels of biological organization, because they are affected by biochemical processes and clearly connected to population and community level effects [32]. To provide an ecologically relevant endpoint for comparison with FA data, four locomotory behavior traits (total distance traveled, proportion of time spent in motion, rate of travel while in motion, and proportion of time spent away from arena edge) were measured using video analysis of individual behavior trials. Behavior was considered a relevant endpoint for this study because  $\text{HgCl}_2$  indirectly affects the nervous system [13]. In addition,  $\text{HgCl}_2$  can become methylated by soil and isopod gut bacteria [14,15], so methylation may contribute somewhat to the toxicity of  $\text{HgCl}_2$ . Methyl Hg can affect behavior and brain function [13].

## CHAPTER 3

### INTRODUCTION

Terrestrial isopods provide a convenient model system for studying the effects of contaminants [1-4] because they are abundant, widespread, easily identified, and have direct contact with numerous soil pollutants [4]. Terrestrial isopods play important roles in decomposition processes such as mechanical and chemical breakdown of leaf litter and enhancement of microbial activity. They are primarily saprophagous, but are also coprophagous and occasionally predatory [5,6]. In addition, they are consumed by a variety of predators such as birds, frogs, lizards, shrews, spiders, ground beetles, scorpions, and chilopods [4]. Their varied diet and wide variety of predators, combined with the fact that many metals (including Hg) bioaccumulate in the hepatopancreas [7], suggest that they may play an important role in trophic transfer of contaminants. In this study, I investigated developmental and behavioral effects of dietary Hg on the terrestrial isopod *Armadillidium vulgare*.

Because soils have a high capacity to sequester Hg, they serve as a sink for atmospheric Hg. Clay soils and organic soil horizons have an especially high affinity for adsorption of atmospherically deposited Hg.  $\text{HgCl}_2$  is one of the most common forms of Hg found across all soil pH ranges [10]. Hg concentrates at relatively shallow depths in the soil [10] where terrestrial isopods are most abundant [4]. Thus, these isopods are well-suited for studying soil Hg contamination. However, few studies have investigated the effects of Hg on isopods. Both chronic exposure to Hg contamination in the field and

acute laboratory exposure to  $\text{HgCl}_2$  were found to correlate with a change in gut bacteria community composition in the terrestrial isopod *Porcellio scaber* [11]. In the aquatic isopod *Asellus aquaticus*,  $\text{HgCl}_2$  inhibited unidirectional influx of Ca and Na [12]. In *in vitro* studies using a variety of other species, mercuric compounds have been shown to inhibit enzyme function affect the immune system, and block cellular receptors and ion channels. By blocking these receptors and channels, mercuric compounds indirectly affect intracellular signaling processes that are important to the nervous system [13].

In this study, I was interested in the effects of chronic exposure to  $\text{HgCl}_2$  via the diet on the symmetry and movement of *A. vulgare*. Fluctuating asymmetry (FA), defined as random deviations from bilateral symmetry, is used as an indicator of developmental instability due to environmental stress in biological organisms [16,17]. FA indices are useful measures of developmental instability for several reasons. First, because comparisons are made within a single individual, development of a pair of bilaterally symmetrical traits is controlled by a single gene complex, controlling for genetic heterogeneity. Second, since two bilaterally symmetrical traits grow at the same time, they grow under homogeneous environmental conditions. Third, prediction of the optimal phenotype can be determined *a priori*, because bilaterally symmetrical traits have an optimal phenotype that has zero asymmetry by definition. Thus, bilateral symmetry facilitates between-site comparisons by controlling for genetic heterogeneity and environmental variation and eliminating the need for preliminary studies to determine the optimal phenotype for each site [9]. Finally, FA is relatively easy and inexpensive to measure [18,19].

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To provide an ecologically relevant endpoint for comparison with FA data, four locomotory behavior traits (total distance traveled, proportion of time spent in motion, rate of travel while in motion, and proportion of time spent away from arena edge) were measured using video analysis of individual behavior trials. Behavior was considered an appropriate endpoint for this study because behavior effectively integrates within-organism changes with population and community dynamics [32]. It was thought that HgCl<sub>2</sub> might affect behavior due to its indirect effects on nervous system function [13], and also because HgCl<sub>2</sub> can be methylated by bacteria found in the gut of terrestrial isopods [14,15], and methyl Hg can affect behavior [13].

## CHAPTER 4

### MATERIALS AND METHODS

#### *Setup*

Specimens of *Armadillidium vulgare* (Brandt 1833) were obtained from a culture of laboratory-reared isopods that had been maintained at the Savannah River Ecology Laboratory for approximately 8 months prior to this experiment. The original brood stock for the culture was obtained from Carolina Biological Supply Co. (NC, USA). After 2 months all original isopods were removed, ensuring that all remaining individuals had been reared under identical conditions. The culture was housed in clear, plastic, containers (32 cm x 18 cm x 13 cm) lined with aspen wood shavings. These containers had lids that snapped on loosely to allow air exchange. A 10 cm x 10 cm plastic Petri dish containing plaster of Paris was provided as a calcium source, and a porous, inverted clay saucer (10 cm diameter x 1 cm high) was provided for moisture and cover. Shelters were kept moist by lightly spraying the top of each with water twice weekly. The culture was maintained on a diet of fish flakes (TetraFin Goldfish Flakes, Tetra, VA, USA). Ambient fluorescent room light was used, so there was not a constant photoperiod. The colony was maintained at a room temperature of approximately 68-72 °C.

*A. vulgare* were separated from the culture's bedding using a Berlese funnel setup with 0.6 mm hardware cloth as a coarse filter and a 60 W bulb as the light and heat source. Adult isopods moved downwards in the funnel, away from the light and heat, while the aspen bedding was retained by the filter. Isopods were isopods were collected

in a plastic box lined with a damp paper towel to prevent desiccation. A large quantity of frass from the colony fell into the collecting container with the isopods. To separate the isopods from the frass, moistened clay shelters (identical to those used in the culture and experiment) were placed on top of the frass overnight. The isopods moved from the frass to the shelters, and were transferred to a separate container by brushing them from the underside of the shelters with a soft brush.

For this experiment, only small isopods were used because I was specifically interested in potential developmental effects. Once the isopods were removed from the culture, they were size sorted by placing them on a container fitted with a fiberglass window screen with 1 mm x 2 mm openings. Ambient overhead fluorescent light was used for size sorting, rather than a Berlese funnel with an incandescent light, in order to protect the larger isopods (those unable to fit through the screen) from heat exposure. After 20 minutes, any smaller isopods clinging to the bottom of the screen were brushed gently into the collection box with a soft brush, and the remaining larger isopods were returned to the colony. The collection box was lined with a damp paper towel to prevent desiccation.

As described above, all individuals used in this experiment were small enough to fit through a 1 mm x 2 mm hole. I noted that mancas (the life stage between emergence from marsupium and first molt) did not appear to respond to the Berlese funnel treatment and seemed to be as likely to walk towards the light as away from it, and none were observed among the sorted isopods. Therefore, all individuals used were young adults that had completed their first molt but were smaller than 2 mm in width.

*A. vulgare* were assigned at random to 12 experimental exposure chambers, with 160 individuals per chamber. Initial mass of isopods was  $0.41 \pm 0.07$  mg (dry weight,  $n = 23$ ). The exposure chambers, calcium source, bedding, clay shelter, and watering schedule were identical to those used for the source culture. Like the source culture, the exposure chambers received ambient room lighting and temperature. While these conditions were not constant throughout the experiment, any changes experienced were the same for all exposure chambers.

### *Hg Exposure*

Hg exposure occurred via dietary intake of  $\text{HgCl}_2$ . Solutions of 0.2 mg/L, 2 mg/L, and 20 mg/L  $\text{HgCl}_2$  were generated by dissolving  $\text{HgCl}_2$  into double de-ionized water. A large batch of each diet was made at the beginning of the experiment and analyzed every seven days to verify Hg content. Diets with nominal concentrations of 0  $\mu\text{g Hg/kg}$  food (control), 600  $\mu\text{g Hg/kg}$  food, 6000  $\mu\text{g Hg/kg}$  food, or 60000  $\mu\text{g Hg/kg}$  food were created by modifying the same type of fish flakes that were fed to the source culture. The low and medium Hg diet levels (600 and 6000  $\mu\text{g Hg/kg}$  food) are realistic for Hg contaminated sites, while the highest Hg diet (60000  $\mu\text{g Hg/kg}$  food) is intentionally high to exaggerate any possible effects on FA or behavioral endpoints [33]. Experimental diets were prepared using a ratio of approximately 900 mg of finely ground fish food spiked with 2.7 mL of either the 0.2 mg/L, 2 mg/L, or 20 mg/L solution. The same ratio was used for the control diet, with double-deionized water instead of an  $\text{HgCl}_2$  solution. Diets were then lyophilized to a constant weight, manually ground, and stored in a desiccator until use.

Each of the 4 treatments was replicated 3 times, so that 3 exposure chambers received control food (C), and 3 received each of the treatment foods (600, 6000, or 60000  $\mu\text{g Hg/kg food}$ ). Food (200 mg) was presented in a small plastic dish and replaced weekly. Total exposure time was 12 weeks. Prior to assessing isopod Hg content isopods were allowed a 48 hour gut purge, then euthanized by freezing, washed in a metal free detergent (Acationox, Baxter Healthcare, MD, USA), and rinsed in double-deionized water. Samples were refrozen and lyophilized in separate glass vials to constant weight.

### *Hg Analysis*

For analyses of total Hg in food or isopod samples, material was analyzed following EPA method 7473, using a DMA80 Direct Mercury Analyzer (Milestone, Inc, Monroe, CT, USA). This method utilizes thermal decomposition, gold amalgamation, with cold vapor atomic absorption detection. Each batch of 12 samples was preceded by a certified reference material of known Hg concentration [Dorm-2 (dogfish muscle), NRCC, Ottawa, Canada], along with a blank. Additional blanks were used periodically to reduce the potential for residue from previous samples due to the high Hg concentration in samples. All reference samples were within the expected range of 4380 to 4900  $\mu\text{g/kg}$  ( $n = 26$ ). Approximately 12 mg of material per sample was used to assess Hg content in the control and low Hg (600  $\mu\text{g Hg/kg food}$ ) diets, while approximately 7 mg of the medium (6000  $\mu\text{g Hg/kg food}$ ) and high Hg (60000  $\mu\text{g Hg/kg food}$ ) diets was used.

Three samples of each diet were analyzed weekly. Isopods from the end of the experiment (day 84) were analyzed for Hg content in pairs, because equipment sensitivity required that samples exceed 2 mg (dry weight) and some individual isopods did not have sufficient mass. A total of 10 individuals per exposure chamber were analyzed, with one exception: only 8 individuals from one of the three low Hg (600 µg Hg/kg food) exposure chambers were analyzed, because a hose came unattached from the DMA80 during analysis of one pair. Individuals used for determining baseline Hg (day 0) were combined until a minimum mass of 2 mg was reached, which required 3 to 7 individuals. A total of 23 individuals from the initial population of small adults were analyzed for Hg content.

The method detection limit (MDL) was calculated using the following formula: 
$$\frac{((\text{mean Hg}_{\text{blanks}})(3 * \text{standard deviation Hg}_{\text{blanks}}))}{(\text{average mass of isopod or diet samples})}$$
. Because three target masses were used in the Hg analysis depending on the type of sample (isopods, high and medium Hg diets (6000 and 60000 µg Hg/kg food), and low Hg (600 µg Hg/kg food) and control diets, three separate MDLs were calculated. Based on an average blank of  $0.1456 \pm 0.0116$  ng of Hg ( $n = 48$ ) and average masses of 0.0039 g (isopods), 0.0078 g (high and medium Hg diets), and 0.0139 g (control and low Hg diets), the method detection limit (MDL) was determined to be 37.02 µg/kg (isopods), 18.66 µg/kg (high and medium Hg diets), and 10.47 µg/kg (control and low Hg diets). Hg content in all food and isopod samples was above the MDL.

Isopods from the end of the experiment (day 84) were analyzed for Hg concentration in pairs, because equipment sensitivity required that samples exceed 2 mg (dry weight) and individual isopods did not have sufficient mass. Individuals used for

determining baseline Hg (day 0) were combined until a minimum mass of 2 mg was reached, which required 3 to 7 individuals.

### *Behavior*

Behavioral trials were conducted in a temperature and humidity controlled environmental chamber, so these characteristics were constant throughout the behavioral trials. Overhead light was provided by four fluorescent light bulbs such that the light intensity reaching the behavioral arenas ( $\pm$  S.E.) was  $82.8 \pm 0.3 \mu\text{mol}/\text{m}^2\text{s}$  ( $n=20$ ). Light intensity was measured using a quantum photosynthetic photon flux light meter (model BQM, Spectrum Technologies, Inc.). The behavioral arenas used were round, plastic Petri dishes with a diameter of 135 mm. Prior to each trial, the arena was wiped with a dilute solution of metal-free soap (Acationox) to remove any residue that may have been left by the previous isopod. Four arenas were set up simultaneously within the environmental chamber. The outside surface of each arena was covered in white lab tape to prevent the isopods from seeing one another.

At the end of the exposure period (day 84), 6 individuals were selected at random from each exposure chamber. For each trial, an individual was placed at the center of each arena. After a 2 min acclimation period, movement was recorded for 5 min using a digital video camera (Panasonic 3CCD). For each trial, one individual from each treatment was used. Isopods were assigned to one of four arenas within the environmental chamber for each trial using a random number generator. Some individuals attempted to climb the wall of the arena and fell off onto their backs. If these behaviors were noted during videotaping, that trial was discarded and repeated with

another individual from the same exposure chamber; however, if these behaviors were not discovered until video analysis, the trial was discarded with no replacement.

Image analysis software packages (Scenalyzer Live v. 4.0 and Virtual Dub v. 1.5.10) were used to prepare video for analysis. The MultiTracker plug-in of ImageJ v. 1.33 was used to automatically identify animal locations and export coordinates. With these coordinates I calculated total distance traveled, proportion of time spent in motion, and rate of travel while in motion. I chose these behavioral traits to focus on possible effects on the overall rate of movement and the consistency of stepwise movement rate. In addition, I used ArcView GIS 3.3 to calculate the proportion of time spent further than 5 mm from the edge of the arena. The edge of the arena was assumed to approximate shelter, as individuals near a wall should be less visible to predators than those in open spaces.

#### *Fluctuating Asymmetry (FA)*

After freezing the isopods, I compared the number of compound eye ommatidia (lenses) between the left (L) and right (R) eyes of each isopod using a dissecting microscope equipped with an external monitor. Counting was done in a conservative manner, meaning that if L and R were found to be different in an individual, the lenses of both eyes were recounted to verify that the difference was not due to human error. The following preliminary tests were carried out to determine whether conditions for FA were met, following the methods described by Palmer [34]. All individuals from week 12 were used for the preliminary tests and subsequent FA calculations, unless ommatidia were uncountable due to excess debris in eye. I tested for directional asymmetry with a  $t$ -

test comparing the departure of the mean (L-R) with the expected departure of zero, and found no significant difference ( $t_{533} = 0.23, p = 0.82$ ) and therefore no evidence of directional asymmetry. The distribution of (L-R) shows a leptokurtic distribution ( $kurtosis = 2.51, p < 0.0001$ ), which indicates a higher central peak than expected under a normal distribution. No skew was evident ( $skew = -0.0615, p = 0.56$ ). These preliminary tests support the use of FA analysis. Leptokurtosis is acceptable and sometimes even expected under the conditions that lead to FA [34-36]. Furthermore, the FA index and statistical test used are both robust to departures from normality [34]. A visual inspection of (L-R) plotted against (L+R)/2 indicated no association between trait size and FA. Previous research with a larger data set indicates that while the number of ommatidia is weakly correlated with body length in *A. vulgare*, the difference between ommatidia counts in the right and left eyes is independent of body size (E.L. Peters, University of Chicago, IL, USA, personal communication). I then calculated an unsigned, size-independent asymmetry index ( $\text{mean}(|L-R|)$ ). All individuals recovered from each chamber were used for this calculation, unless I was unable to count ommatidia due to damage or excessive debris in eye. The number of individuals used for FA calculations from each exposure chamber varied from 26 to 56, with a mean sample size of  $44.5 \pm 2.3$ . In addition, these individuals were sexed after counting ommatidia. Sex was included in the statistical model to account for any possible effects of sex on FA levels.

### *Statistics*

Statistical analyses were performed using SAS 9.0. The experimental setup followed a nested design, where the exposure chamber was the statistical unit of interest.

FA data were analyzed with a modified Levene's test, using a nested one-way ANOVA of |L-R| [34]. All remaining analyses (isopod mass, Hg concentration in isopods and diets, and all behavioral metrics) used a one-way ANOVA of the measurement of interest. Differences were considered significant at  $p < 0.05$ . When appropriate, multiple comparisons were carried out using Tukey's HSD.

Because of the low number of replicates per treatment level, low statistical power was a concern. To reduce the chance of finding no difference among treatments when a difference did exist, data from replicate exposure chambers were pooled for use in a second one-way ANOVA with individual isopod as the unit of interest. Pooling was only undertaken if no significant difference was observed in the initial nested ANOVA or initial multiple comparisons, and only considered appropriate when no difference was found among replicate exposure chambers ( $p > 0.25$ ) [37]. Using these criteria, pooled data were analyzed for final mass, FA index, total distance traveled, proportion of time spent in motion, and median rate of travel while in motion. Where pooling was done, statistics for both pooled and non-pooled analyses are presented.

## CHAPTER 5

### RESULTS

Mean measured concentrations of Hg in control, low Hg (600  $\mu\text{g Hg/kg food}$ ), medium Hg (6000  $\mu\text{g Hg/kg food}$ ), and high Hg (60000  $\mu\text{g Hg/kg food}$ ) across all weeks were  $49.90 \pm 0.97 \mu\text{g/kg}$ ,  $524.89 \pm 4.95 \mu\text{g/kg}$ ,  $6147.85 \pm 57.91 \mu\text{g/kg}$ , and  $65744.02 \pm 830.59 \mu\text{g/kg}$  respectively ( $n=36$ ). Hg did not volatilize, so concentrations remained the same from the beginning of the experiment to the end (appendix A).

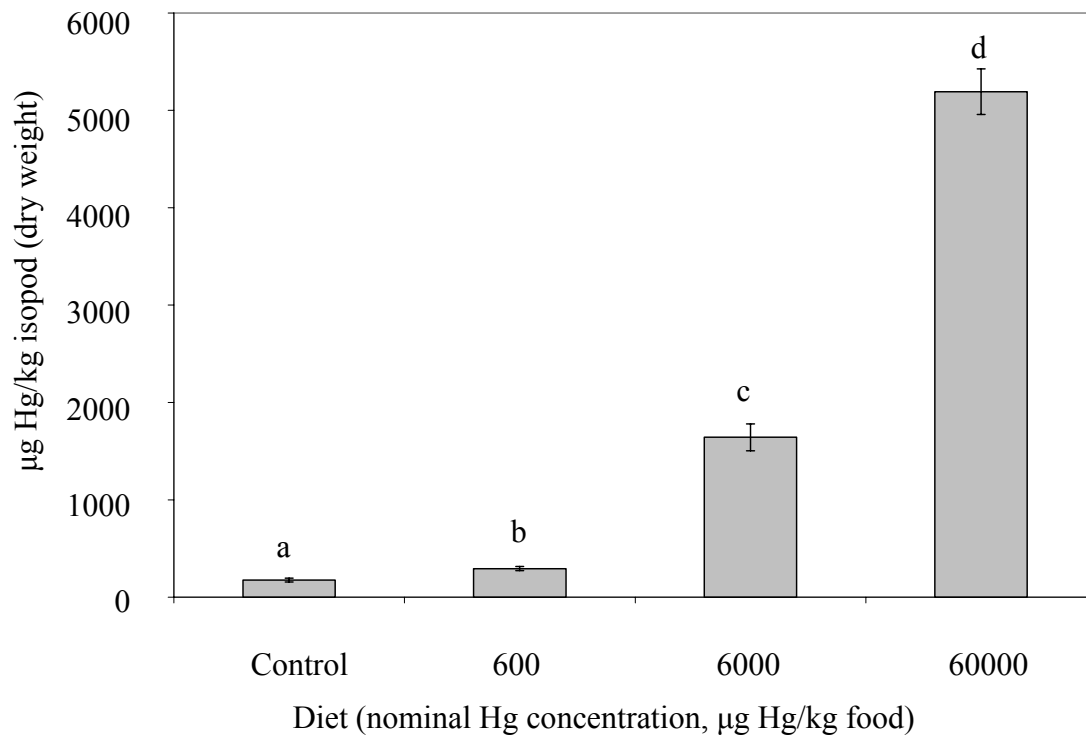
Hg concentration in isopods after 12 weeks exposure increased with increasing concentration of Hg in diet, with log transformed Hg body burdens differing significantly among isopods receiving all diets (Figure 1;  $f_{3,8} = 377.80$ ,  $p < 0.0001$ ). After 12 weeks exposure, final individual dry mass did not differ among containers (control:  $1.85 \pm 0.18$  mg; low Hg diet (600  $\mu\text{g Hg/kg food}$ ):  $2.01 \pm 0.22$  mg; medium Hg diet (6000  $\mu\text{g Hg/kg food}$ ):  $1.97 \pm 0.14$ ; high Hg diet (60000  $\mu\text{g Hg/kg food}$ ):  $2.25 \pm 0.16$  mg; nested:  $f_{3,8} = 0.87$ ,  $p = 0.49$ ; pooled:  $f_{8,47} = 0.77$ ,  $p = 0.63$ ). After 12 weeks, individual mass in all treatment groups increased to approximately five times the initial mass of  $0.41 \pm 0.07$  mg (dry weight)). The overall mean percentage of individuals recovered ( $\pm$  S.E.) from the original population of 160 individuals per container was  $29.4 \pm 1.3\%$ . In one control chamber only 18.8% of the individuals were recovered, which was much lower than the percentage recovered from any other chamber. This recovery percentage was a statistical outlier. Removal of this outlier changed the mean percentage recovered across all treatments to  $30.3 \pm 0.9\%$ . There was no difference in the mean recovery percentage

among treatments (control:  $30.6 \pm 1.9\%$  (outlier excluded), low Hg (600  $\mu\text{g Hg/kg}$  food):  $32.2 \pm 1.9\%$ , medium Hg (6000  $\mu\text{g Hg/kg}$  food):  $27.5 \pm 2.0\%$ , high Hg (60000  $\mu\text{g Hg/kg}$  food):  $31.0 \pm 1.8\%$ ;  $f_{3,7} = 1.21$ ,  $p = 0.37$ ).

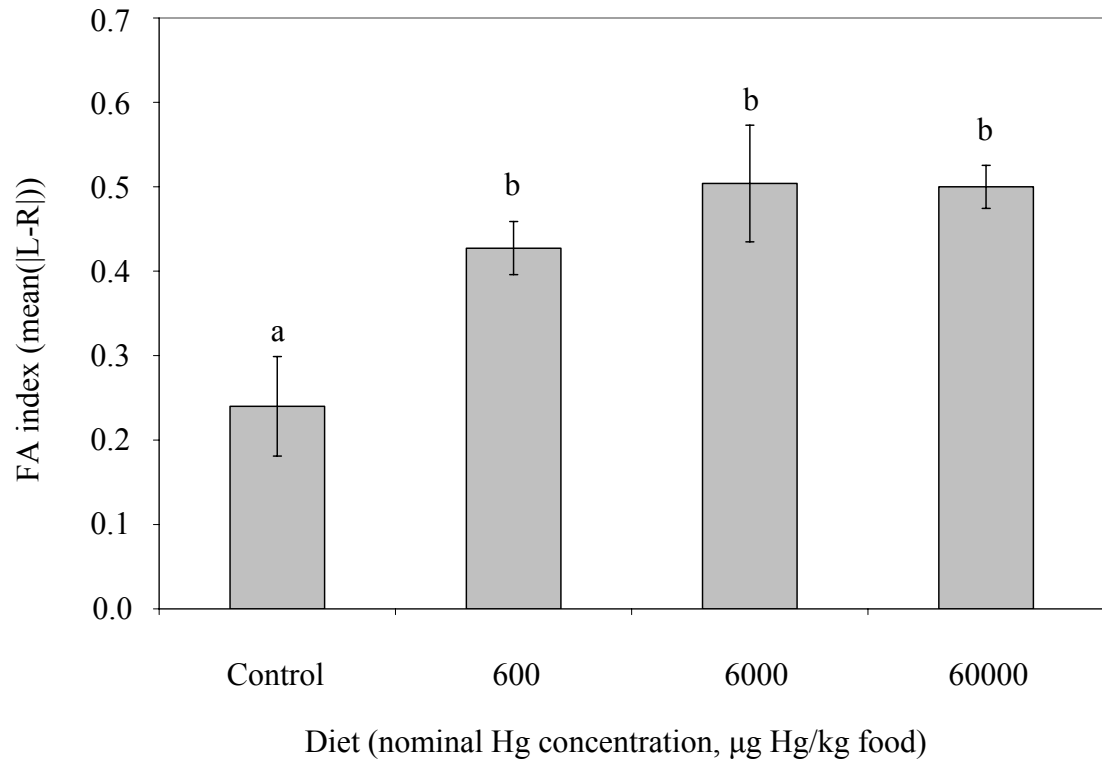
Ommatidia number was more asymmetrical in Hg-exposed populations than in controls ( $f_{3,8} = 5.66$ ,  $p = 0.0223$ ). However, there was no evidence that the magnitude of asymmetry ( $|L-R|$ ) differed among the three Hg diets (Figure 2). Because no difference was found among experimental units at the  $p = 0.25$  level ( $f_{8,522} = 0.77$ ,  $p = 0.63$ ), data were pooled to increase statistical power for multiple comparisons [37]. Pooling data resulted in no additional significant differences.

Hg ingestion had no significant effect on behavior (Figure 3), including total distance traveled (nested:  $f_{3,7} = 0.77$ ,  $p = 0.54$ ; pooled:  $f_{3,49} = 0.97$ ,  $p = 0.41$ ) proportion of time spent in motion (nested:  $f_{3,7} = 0.52$ ,  $p = 0.52$ ; pooled:  $f_{3,49} = 0.55$ ,  $p = 0.65$ ), median rate of travel while in motion (nested:  $f_{3,7} = 1.33$ ,  $p = 0.34$ ; pooled:  $f_{3,49} = 1.55$ ,  $p = 0.19$ ), and proportion of time spent further than 5 mm from the edge of the arena ( $f_{3,7} = 0.63$ ,  $p = 0.62$ ). These data exclude behavioral trials from one of the three control replicate exposure chambers, due to extreme behavioral differences between two of the three isopods from that chamber and those from the other two control chambers. First, mean individual total distances ( $\pm$  S.E.) within the other two control chambers were  $1.7 \pm 0.3$  m/5 min ( $n = 4$ ) and  $1.7 \pm 0.1$  m/5 min ( $n = 5$ ), compared with a mean individual total distance of  $2.3 \pm 0.5$  m ( $n = 3$ ) for the unusual control. Total distance for the unusual control as a whole did not differ significantly from the other two controls (one-way ANOVA, among chambers of the same treatment,  $f_{8,44} = 1.32$ ,  $p = 0.30$ ), but one individual from the unusual control moved 3.4 m in 5 min and was an outlier. Second,

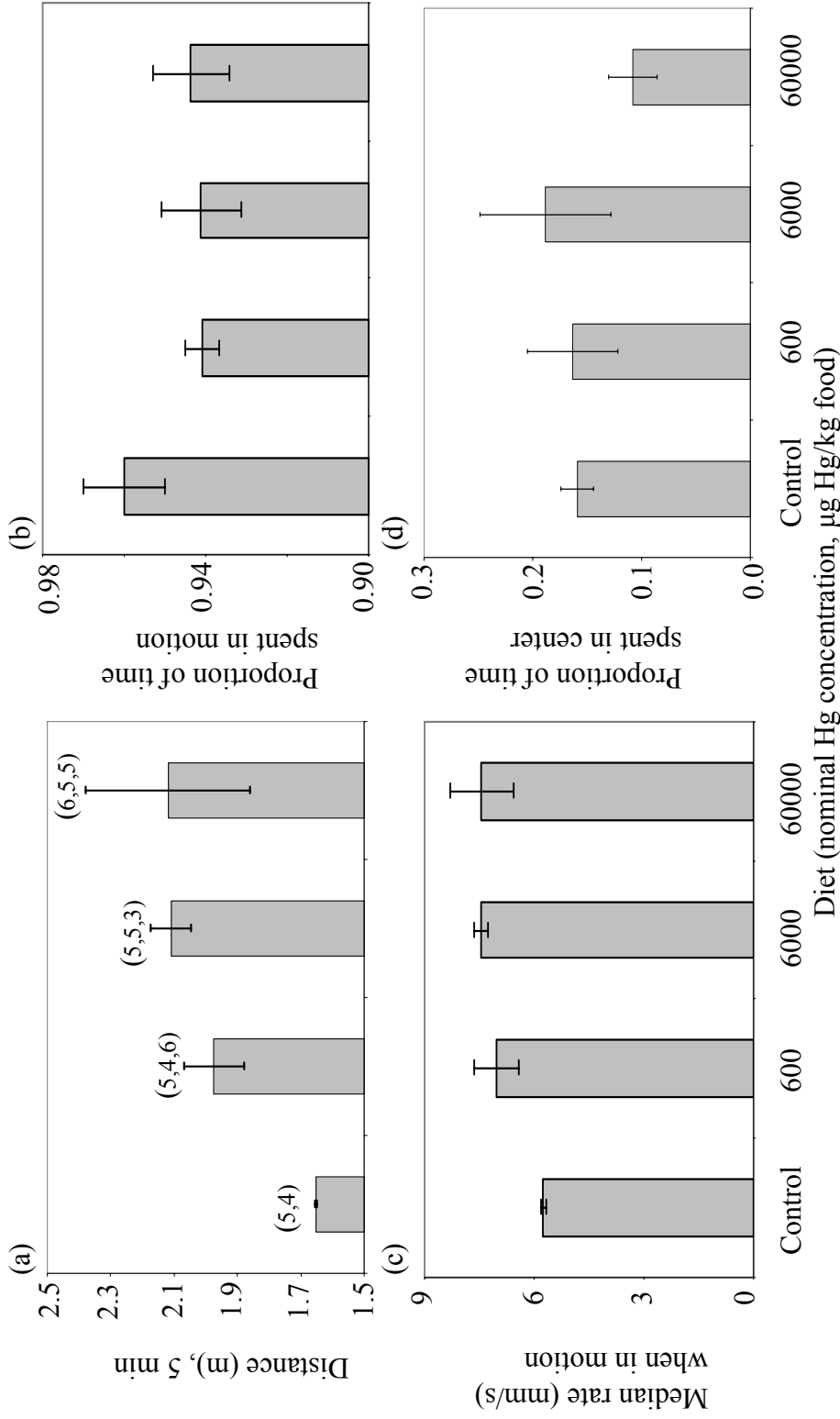
the mean proportion of time spent  $> 0.5$  cm from the edge of the arena ( $\pm$  S.E.) for the unusual control was  $0.64 \pm 0.17$  ( $n = 3$ ), whereas the proportions of time spent  $> 0.5$  cm from the edge for the other two controls were  $0.14 \pm 0.07$  ( $n = 4$ ) and  $0.17 \pm 0.07$  ( $n = 5$ ). Not only was the proportion of time spent in the center significantly higher for the unusual control than for the other two (one-way ANOVA, among chambers of the same treatment,  $f_{8,44} = 4.13$ ,  $p = 0.001$ ), one individual (not the same individual as the total distance outlier) spending 98% of its trial  $> 0.5$  cm from the edge of the arena. Third, while turn rate and turn angle were not tested for in this experiment, a visual inspection of the movement paths of these individuals revealed a pattern of frequent small circles, as opposed to the occasional gradual turns in individuals seen in the movement paths of individuals from all other chambers. In addition to the unusual behavior of two of the three individuals used for behavior trials from the unusual control, this chamber was the same control chamber that was a statistical outlier in terms of percent recovery of individuals from the beginning to the end of the experiment, suggesting higher mortality than all other exposure chambers at all treatment levels. Results that include behavioral trials from the omitted control chamber are presented in appendix B



**Figure 1.** Mean Hg concentration ( $\pm$  S.E.) of individual *Armadillidium vulgare* after 12 weeks exposure to  $\text{HgCl}_2$  contaminated food or control food. Isopods were analyzed in pairs in order to meet minimum mass requirement for analytical method ( $n = 5$  pairs for all chambers, exception  $n = 4$  pairs for one chamber from low Hg diet ( $600 \mu\text{g Hg/kg}$  food) due to mechanical malfunction during analysis). Treatments with different letters above the column had significantly different FA levels ( $p < 0.05$ ). Initial Hg concentration (week 0) was  $157.58 \pm 22.69 \mu\text{g Hg/kg}$  isopod dry weight ( $n = 4$  samples; 23 isopods total).



**Figure 2.** Fluctuating asymmetry (FA) index ( $\pm$  S.E.). L and R are the number of ommatidia per eye on the left and right sides of the head of *Armadillidium vulgare*. N = 3 exposure chambers per diet. The number of individuals per exposure chamber differed for each chamber (control: 26, 42, 47; low Hg diet (600 µg Hg/kg food): 43, 47, 56; medium Hg diet (6000 µg Hg/kg food): 38, 42, 47; high Hg diet (60000 µg Hg/kg food): 42, 49, 55). Treatments with different letters above the column had significantly different FA levels ( $p < 0.05$ ).



**Figure 3.** Results of 5 minute behavioral trials in a 135 mm circular arena (control: n = 2, Hg diets: n = 3; 3 number of subsamples (individuals) per replicate is shown in parentheses above the columns in (a) and is the same for all four figures). (a) Distance traveled in 5 minutes ( $\pm$  S.E.). (b) Proportion of time with distance per frame (6 frames = 1 s)  $>$  0 ( $\pm$  S.E.). (c) Median rate of travel when distance per frame  $>$  0 ( $\pm$  S.E.). (d) Proportion of time further than 5 mm from edge of arena ( $\pm$  S.E.). One control chamber is omitted (see text). No differences are statistically significant ( $p >$  0.05).

## CHAPTER 6

### DISCUSSION

As predicted, ommatidia number was more asymmetrical in populations of *A. vulgare* that were fed HgCl<sub>2</sub> than in control populations, indicating reduced developmental stability in Hg exposed *A. vulgare*. This asymmetry in the laboratory-reared isopods is consistent with the results of Vilisics et al. [17], who found that FA was measurable by ommatidia number in isopods, and with E.L. Peters et al. (University of Chicago, IL, USA, personal communication), who found that Hg concentration was correlated with the degree of FA in ommatidia number in wild populations of *A. vulgare*.

The most common hypothesis to explain the increase of FA with stress is that because energy availability is limited, the energy used to compensate for a stressor reduces the amount of energy available for regulation of stable development, resulting in asymmetry [9,22,38-40]. Because terrestrial isopods compensate for Hg intake by storing Hg in the hepatopancreas rather than excreting it [7], the energy limitation hypothesis suggests that the energy required to store Hg in the hepatopancreas may have contributed to increased FA in the ommatidia of isopods receiving Hg diets. There is evidence that for some, but not all, species of terrestrial isopods, storage of Hg in the hepatopancreas is an energetically expensive process [8]. Because food was given in excess in this study, energy limitation was less likely to be a factor in FA than in field populations where food may be limited. However, many species are able to detect and avoid some contaminants in food, and previous research suggests that this may be true of terrestrial isopods with

certain contaminants [8]. Further research is needed to determine if *A. vulgare* can detect and avoid Hg contaminated food. Avoidance of contaminated food would limit energy availability, even when food is given in excess. If the energy limitation hypothesis is correct, a future study using restricted amounts of food may result in higher levels of FA than those seen in this study. A second hypothesis that is less prevalent in the literature states that FA may result from disruption of internal cellular development [9], such as direct effects of a toxic chemical. As mercuric compounds can interfere with processes such as enzyme, ion channel, nervous, and immune functions, it is possible that ommatidia development was directly affected by the toxic effects of HgCl<sub>2</sub>. These hypotheses are not mutually exclusive, so it is possible that FA in this study could have been caused by both indirect energy limitation due to Hg storage and by the direct toxic effects of Hg.

While FA was higher in *A. vulgare* receiving Hg diets than in those receiving the control diet, no significant correlation was found between FA and Hg concentration among the three non-control diets in this study. This lack of correlation suggests that FA of ommatidia in *A. vulgare* may be a more sensitive indicator of soil Hg at lower concentrations than at those used in this study, in which saturation of the effect apparently occurred. Further study with lower concentrations of Hg is needed to obtain a more precise dose-response relationship between FA and Hg concentration. Also, exposing isopods to Hg beginning at the manca stage would expose them for a longer period of time during development, which might increase the sensitivity of the test. As growth slows, the addition of ommatidia also slows, so the use of large, slow-growing adults is not recommended for FA studies using ommatidia.

Ecotoxicological studies are often criticized for a lack of ecological relevance, particularly in terms of linking effects at the individual and within-organism levels of organization with effects at population, community, and ecosystem levels [e.g. 31]. Behavioral studies are effective integrators of levels of biological organization, because they are affected by biochemical processes and clearly connected to population and community level effects [32]. To incorporate ecological relevance, I investigated whether there was a correlation between FA and locomotory behavior in *A. vulgare*. Specifically, I was interested in total distance traveled, proportion of time spent in motion, rate of travel while in motion, and proportion of time spent near the edge of an arena.

Unlike FA, there was not a statistically significant correlation between Hg concentration and any of the behavioral traits mentioned. However, some potentially biologically significant trends are apparent. In particular, total distance traveled initially increased with increasing Hg concentration. In addition, control isopods appeared to spend more time in motion than those receiving the three Hg diets. When isopods were in motion, those from the control may have moved at a slower rate than those receiving Hg diets. Combined, these three observations suggest that the control isopods traveled at a slow, steady pace throughout the 5 minute behavioral trials, whereas those receiving Hg diets may have traveled faster but more intermittently. An additional change observed is that isopods receiving the highest Hg diet appeared to stay near the arena edge more often than those receiving the control and two lower Hg diets.

The lack of statistically significant increases in total distance traveled with response to increasing Hg ingestion may have been due to low sample size.

Alternatively, the lack of significant differences in behavior, as well as mean mass, may be related to the role of the hepatopancreas in terrestrial isopods. The hepatopancreas binds metals as granules, isolating them from the rest of the body. Direct toxicity in isopods occurs when the receptors of the hepatopancreas are saturated or when contaminant intake rate exceeds the rate of granule formation [7]. Therefore, it may be that behavior and mass were not affected by Hg because the hepatopancreas had additional storage capacity. On the other hand, it is possible that HgCl<sub>2</sub> has no effect on body size or the behavioral traits measured in this study. Behavior was chosen as an endpoint for this study due to its effectiveness as an integrator across levels of biological organization [32], and because a possible link was supposed due to the indirect effects of HgCl<sub>2</sub> on the nervous system [13]. Finally, any behavioral changes in isopods associated with HgCl<sub>2</sub> may have been difficult to identify due to the general nature of the behavioral endpoints used. In future studies, the use of more ecologically relevant endpoints with a specific stimulus and specific optimal response may be more useful in determining whether HgCl<sub>2</sub> has an effect on behavior. Examples include predator avoidance, shelter use, and phototaxis. In addition, significant differences in more ecologically relevant endpoints would provide a more meaningful connection between FA and fitness or population dynamics.

Individuals from one control chamber from this experiment showed unusual behavioral patterns, such as traveling a longer distance than other individuals, spending an unusually long time away from the edge of the arena, and moving in frequent small circles rather than occasional gradual turns. This same control chamber had a lower percent recovery of individuals after 12 weeks than all other exposure chambers from all

treatment levels. This exposure chamber was set up and maintained in the same location and same manner as all other exposure chambers, and the individuals used came from the same culture as those from all other chambers. The frequent small circles and rapid movement seen in the behavior trials indicate the presence of a pathogen or additional contaminant not present in the other containers. Interestingly, FA in the population of isopods from this chamber was intermediate between the other control chambers. This may suggest that whatever abnormal conditions existed in this chamber occurred late in the experiment, such that most ommatidia had already formed by the time behavior and mortality were affected. Alternately, the source of the unusual behavior and high mortality may have been a variable that has no effect on physical developmental instability.

There is evidence that while meristic traits such as ommatidia number can serve as indicators of asymmetry, they are not as sensitive as metric traits such as lengths of leg or antenna segments [30,41]. In particular, Velisics et al. argued that ommatidia number was less sensitive for use in FA than the other traits included in that study, which were metric traits [30]. This difference in sensitivity may be because a stress threshold must be met for each step-wise difference in a meristic trait, whereas metric traits can vary subtly and continuously with small differences in stress. Because counted traits are a less sensitive indicator of asymmetry, a greater sample size is needed [42]. However, the requirement for a larger sample size is balanced by the fact that counting meristic traits requires less training and equipment than measuring metric traits. Using meristic traits is therefore less expensive and allows for a larger sample size. Furthermore, both intra- and inter-observer error is a large concern with metric traits, so metric traits must be

measured multiple times to minimize measurement error [43,44]. In contrast, meristic traits that are easy to count can be used with minimal observer error [18]. In addition, if measured traits such as limbs or antennae are used for isopod FA studies, there is a chance that parts of these traits will have been lost and regenerated in the past, which would result in asymmetry [30]. In general, the use of multiple traits is preferable over the use of single traits in FA studies [45]. However, if limited time, money, expertise, and equipment are available, and only one trait can be measured, there are advantages to using an easily counted trait such as ommatidia number for FA studies.

The purpose of this study was to see if FA could be induced in terrestrial isopod ommatidia by dietary intake of  $\text{HgCl}_2$ . Ingestion of  $\text{HgCl}_2$  resulted in an increase in FA, which supports the use of isopod ommatidia in future FA studies, such as biomonitoring of Hg cleanup efforts. A secondary purpose was to determine if there was a correlation between FA effects and effects on a more ecologically relevant trait. Behavior was chosen for this because behavior is an effective integrator of different levels of biological organization. The behavioral traits measured did not result in any statistically significant differences, so no correlation between FA and behavior could be confirmed. However, there are some potentially biologically significant trends apparent in the diet. Combined, these trends suggest that isopods receiving control diets traveled at a slower, more consistent pace than those receiving  $\text{HgCl}_2$ . Further behavioral studies with larger sample sizes are needed to further investigate these trends.

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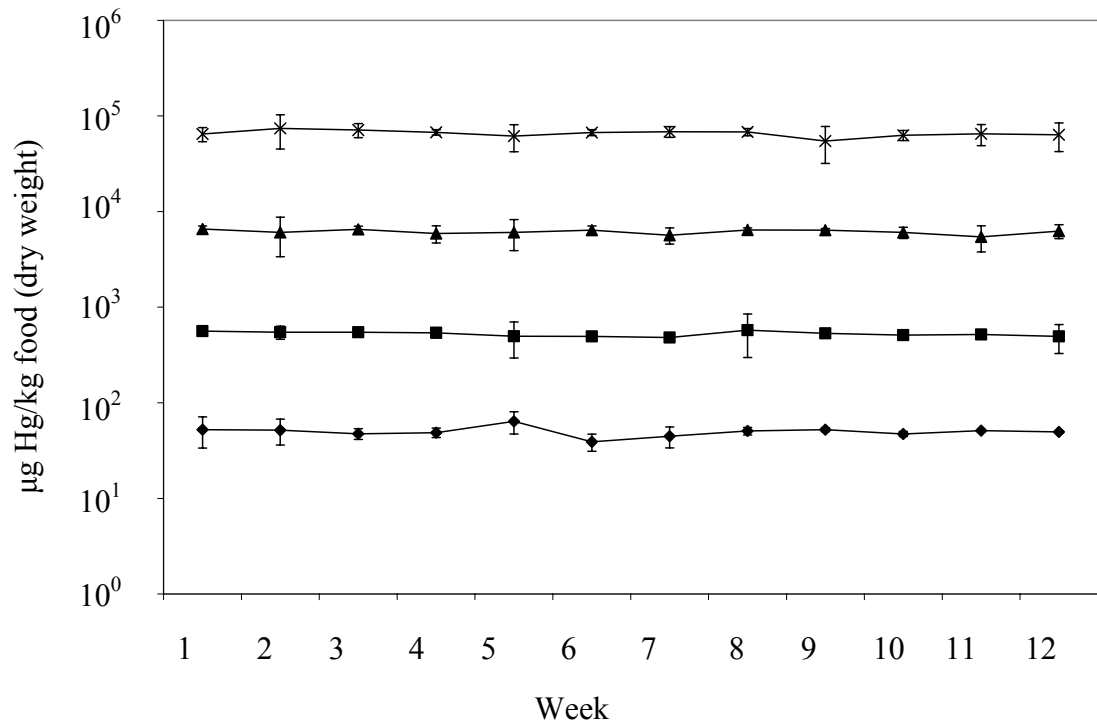
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## APPENDIX A

### WEEKLY HG CONCENTRATION IN DIETS

Hg can easily volatilize, so the possibility of declining Hg concentration over time was a concern. Because of this, Hg concentration each diet was analyzed weekly (n=3) for the duration of the 12 week experiment. No difference was observed in log transformed Hg concentration between week 1 (start) and week 12 (final) for any diet (control:  $t_{2,03} = 0.65, p = 0.58$ ; low Hg (600  $\mu\text{g Hg/kg food}$ ):  $t_{2,03} = 1.74, p = 0.22$ ; medium Hg (6000  $\mu\text{g Hg/kg food}$ ):  $t_4 = 1.12, p = 0.33$ ; high Hg (60000  $\mu\text{g Hg/kg food}$ ):  $t_4 = 0.23, p = 0.83$ ), indicating that Hg concentration remained constant throughout the duration of the experiment. Actual weekly and overall concentration of each diet can be seen in Figure 4.



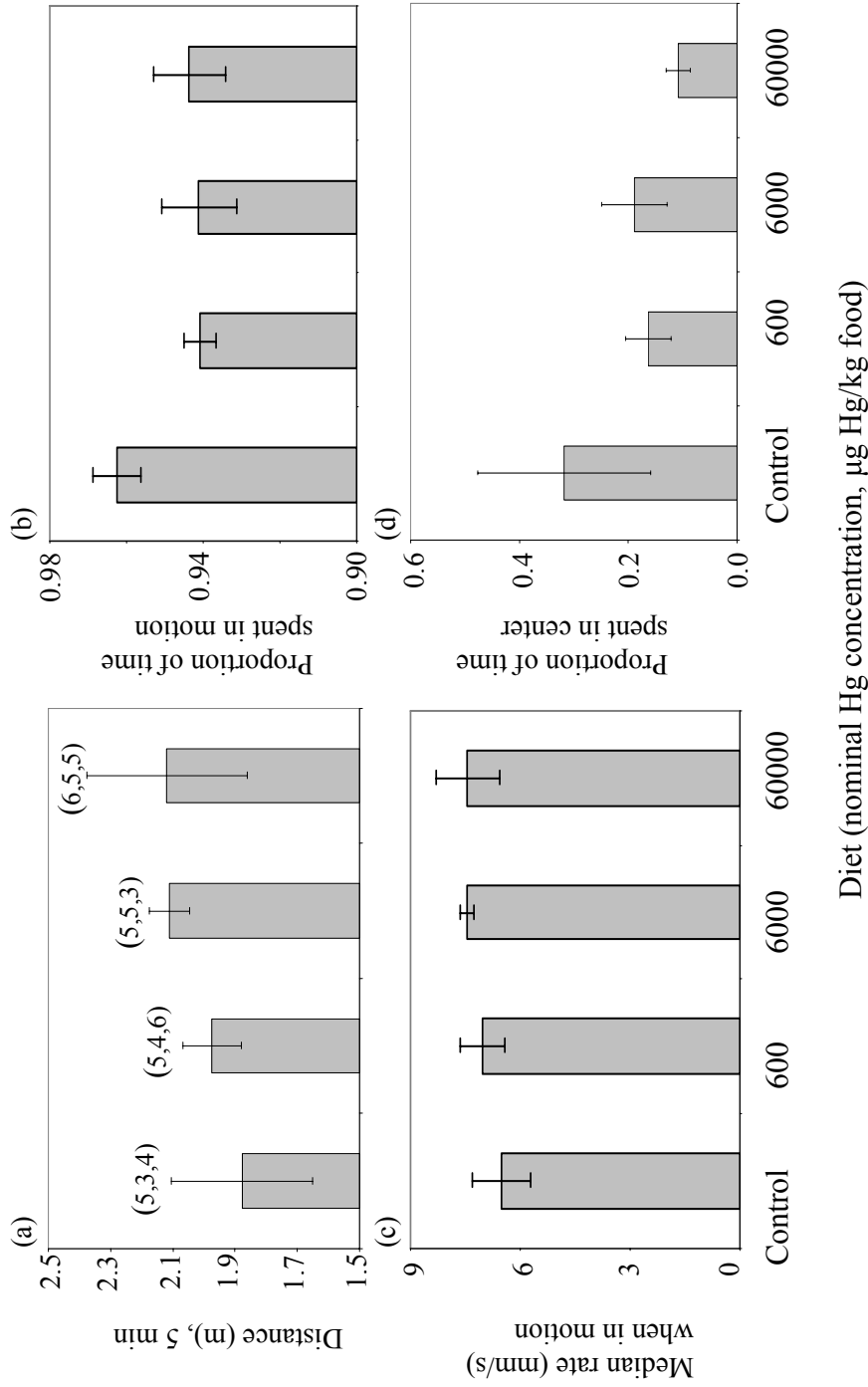
**Figure 4.** Weekly concentration of Hg in diets. N = 3 samples per week for each diet. Error bars denote 95% confidence interval. (nominal concentrations: × = 60000 µg Hg/kg food, ▲ = 6000 µg Hg/kg food, ■ = 600 µg Hg/kg food, ◆ = control).

## APPENDIX B

### RESULTS FROM ALL BEHAVIORAL TRIALS

Because isopods from one control chamber showed very different behavior patterns than those from the other two control chambers (see Chapter 5 for full description), this chamber was omitted from analysis in the main body of this thesis. Analysis of all behavioral trials, including those from the unusual control chamber, is presented here.

Hg ingestion had no significant effect on behavior (Figure 5), including total distance traveled (nested:  $f_{3,8} = 0.24, p = 0.86$ ; pooled:  $f_{3,52} = 0.44, p = 0.72$ ) proportion of time spent in motion (nested:  $f_{3,8} = 1.46, p = 0.30$ ; pooled:  $f_{3,52} = 0.88, p = 0.46$ ), median rate of travel while in motion (nested:  $f_{3,8} = 0.41, p = 0.75$ ; pooled:  $f_{3,52} = 0.82, p = 0.49$ ), and proportion of time spent within 0.5 cm of the edge of the arena ( $f_{3,8} = 1.18, p = 0.38$ ).



**Figure 5.** Results of 5 minute behavioral trials in a 135 mm circular arena, all data (n=3; number of subsamples (individuals) per replicate is shown in parentheses above the columns in (a) and is the same for all four figures). (a) Distance traveled in 5 minutes ( $\pm$  S.E.). (b) Proportion of time with distance per frame (6 frames = 1 s)  $>$  0 ( $\pm$  S.E.). (c) Median rate of travel when distance per frame  $>$  0 ( $\pm$  S.E.). (d) Proportion of time further than 5 mm from edge of arena ( $\pm$  S.E.).

## APPENDIX C

### PRELIMINARY INVESTIGATION

A preliminary investigation occurred prior to the main study described in this thesis, to develop and verify the methods that would be used in the main study. The preliminary study was a joint effort of Steven Harper, Eric Peters, Ashley Hayes, Heather Brant, and Carol Flaute. The results and observations from the preliminary investigation that contributed to the design of the main study are presented below. The information presented here is given in a general descriptive manner in order to provide insight into the experimental design choices made for the main study and to aide future researchers.

#### *Comparison of materials and methods to those of main study*

This section is not a comprehensive account of the materials and methods of the preliminary investigation. Instead, it is a description of the main differences between the two studies. Only those methods and differences which specifically impacted the design of the main study are included. A description of the methods of the main study is located in chapter 4 of this thesis.

The isopods used in the preliminary investigation were obtained from the same culture as those from the main study, but they were large, slow-growing adults that were several times larger than the small, fast-growing adults used in the main study. Only the control and highest Hg (60000 µg Hg/kg food (nominal concentration)) diets described in the main study were used in the preliminary investigation. Isopods were fed 500 mg

weekly for 9 weeks. Isopod Hg body burdens were analyzed periodically using 10 individuals from each exposure chamber to determine if Hg concentration in isopod tissues continued to increase over time or if a saturation point was reached. Fluctuating asymmetry and Hg analysis were measured in the same manner as in the main study.

Behavioral trials consisted of a 2 minute acclimation period followed by a 5 minute trial, as in the main study. However, the methods used were different. In contrast to the main study, in which the acclimation period occurred under the same conditions as the actual trial, the acclimation period of the preliminary investigation took place under an inverted plastic film canister (used for 35 mm camera film) that was placed in the center of the arena and attached to a string via a hole drilled in the center of the canister. When the 5 minute trial began, the canister was lifted away remotely. The arena for the preliminary investigation was 39 cm in diameter, and was made of a textured white plastic. The intersection of the floor and wall of the arena was a gradual, rounded transition. Behavioral trials for the preliminary study occurred in the same environmental control chamber as the main study. Each individual isopod was used for 2 trials: one under the same white fluorescent lights as the main study, and the other under red incandescent lights. This comparison of red and white lights was to determine whether light conditions would affect movement patterns. It was thought that the red light might be perceived as closer to the dark conditions in which terrestrial isopods are typically found. Finally, once position coordinates were determined as described in chapter 4, the Animal Movement Extension of Arcview 3.x was used. This software calculates the following movement statistics: minimum distance, maximum distance, total distance,

number of bearings, mean bearing, R concentration of angles, angular deviation, linearity, primary axis length, secondary axis length, primary axis angle, and eccentricity.

*Relevant results and discussion of their impact on the main study*

Food was presented to each exposure chamber weekly in 500 mg servings. The majority of this food was not consumed, so the serving size was reduced to 200 mg per week for the main study. Hg body burden continued to increase in the Hg treatment over the 5 weeks of the preliminary study, with no evidence of saturation (Figure 6). My original intent for the main study was to set the duration of exposure based on the length of time that was required for body burden saturation to be reached; however, as body burden continued to increase throughout the preliminary study, the exposure duration of the main study was set longer than that of the preliminary study.

Fluctuating asymmetry did not differ between isopods receiving the control and Hg diets in the preliminary study ( $X^2$  test (contingency table analysis),  $p = 0.78$ ). This was as expected, because the individuals used in the preliminary study were large and slow-growing, allowing for very few physical developmental changes to occur. The preliminary study supported the idea that individuals in the main study should be as young as possible, to take advantage of the potential for developmental effects during the rapid-growth phase.

The software used for the behavioral analyses (the Animal Movement Analysis extension of Arcview) was designed for movement analysis of field populations of animals. We used it for the preliminary investigation to see whether its output could be meaningfully interpreted for movement analysis within a circular behavioral arena.

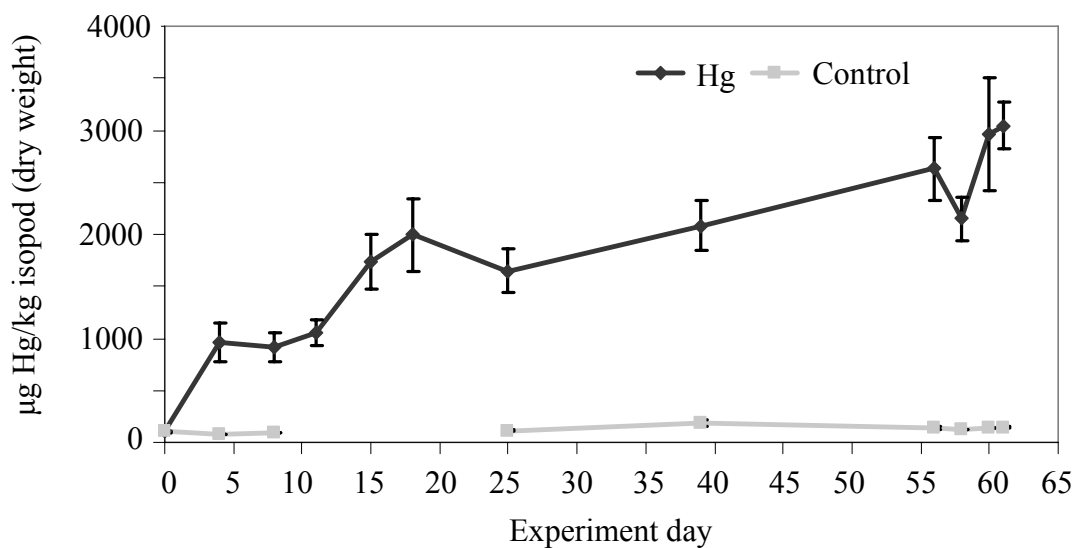
Based on our experiences during analysis of the preliminary behavioral trials, we decided that this software was not appropriate for this study. The Animal Movement Analysis extension may be useful for arena-based behavioral studies when the organism's behavioral path is small enough to remain unconstrained by the edge of the arena. However, most isopods in the preliminary study (and later in the main study) primarily ran in circles at the outer limits of the arena with only occasional ventures into the center, and most of the metrics analyzed by the Animal Movement Analysis extension are measures of the overall shape and spread of the outer limits of the movement path, so for most individuals the results provided by the software simply indicated the size and circular shape of the arena. For the main study, I directly calculated stepwise distances from XY coordinates rather than using the Animal Movement Analysis extension.

One purpose of the preliminary behavior trials was to determine if the type and color of light influenced individual behavior. In addition to finding no mathematical difference between the total distances traveled by the same individual in the red-light and white-light two trials (paired *t*-test,  $p > 0.05$ ), I visually compared the red-light and white-light movement paths for each individual. I was looking for noticeable differences in movement shape, and expected only generally similarities. Instead, I found that many individuals had red-light and white-light movement paths that were nearly duplicates of each other. This surprising observation suggested that light color and type used did not seem to affect behavior patterns, and that each individual had some internal predisposition towards a certain movement pattern. As video analysis was much more difficult under red light than under white light because of low variation in pixel color under red light,

and movement patterns were so similar between trials of the same individual, I decided to use white light in the main study.

A major problem in the behavioral trials of the preliminary investigation was that numerous individuals flipped on their backs during the behavioral trials and were unable to right themselves. This occurred due to one of two causes. First, during the acclimation period, many individuals crawled onto the inside walls of the inverted film canister. When the canister was lifted remotely, these individuals were jostled out of the canister and traveled through the air, often landing on their backs. To eliminate this problem, I did not use the film canisters for the acclimation period of the main study, but instead let the isopods roam under the same conditions as the behavioral trial. The film canisters were originally used to keep the isopod in the center of the arena until the beginning of the trial, but I also had concerns that the rapid change from darkness during the acclimation period to light for the trials would unnecessarily startle and stress the isopods if the film canister was used.

Second, because the surface of the arena was textured, and the junction between the wall and floor was a gradual, rounded incline, the isopods were easily able to climb the sides of the arena. Many of those that climbed the sides either fell off onto their backs, or continued to climb until they had completely exited the arena. I attempted to correct this problem by using plastic Petri dishes in the main study, as Petri dishes are more slippery than the preliminary trial arena and the intersection of the floor and wall of a Petri dish is a right angle. However, some individuals in the main study were still able to climb the wall of the Petri dish, so while this problem was greatly reduced by changing the arena, it was not completely eliminated.



**Figure 6.** Mean Hg concentration ( $\pm$  S.E.) of individual *Armadillidium vulgare* from preliminary investigation. Isopods were fed either a diet of  $\text{HgCl}_2$  (60000  $\mu\text{g Hg/kg}$  food) or a control diet.  $N = 3$  exposure chambers, 10 individuals per chamber per sample. Isopods were combined in analysis in order to meet a minimum mass of 4 mg.

## APPENDIX D

### SUGGESTED DIRECTIONS FOR FUTURE RESEARCH

This appendix contains two types of suggestions for future research related to this study. Suggestions of areas where the methodology used in this study can be improved and suggestions of potential topics for future related studies are included. Suggestions are grouped according to topic.

#### *Food*

In this study, food was provided to exposure chamber populations weekly. By the end of each week, the food had invariably molded. To avoid mold, it would be better to feed several times a week, or feed weekly but remove the food after a shorter period of time.

The control diet in this study contained some Hg, even though no Hg was added. This may be due to the choice of fish flakes as the basic food source. Future Hg research should explore the use of different foods with a lower Hg content.

As described in chapter 6, the most common hypothesis given to explain fluctuating asymmetry involves energy limitation. In this study, food was given in excess. I would like to suggest a future study using a limited food source, to see if this exacerbates the effect of HgCl<sub>2</sub> on FA. Using a limited food source may more realistically approximate conditions found in natural ecosystems, where food is often limited. As food avoidance may limit energy availability even when food is given in

excess, future research should investigate whether *A. vulgare* are able to detect and avoid Hg contaminated food. This would require a food source that does not absorb moisture and mold, so that initial and final mass of food could be compared among treatments. Research on the effects of combinations of HgCl<sub>2</sub> with other contaminants would also be useful.

#### *Exposure chamber maintenance and mortality*

Mortality was high across all treatment groups in this study, including the control. On average, only 30% of the individuals that were initially introduced to each exposure chamber were recovered at the end of the experiment (chapter 5). This may have been caused by many factors. Initial densities may have been too high. The loosely fitting lids of the exposure chambers may have allowed too much moisture to escape. Aspen bedding may not be the optimal bedding material. These concerns should be investigated before undertaking future studies. In addition, populations may have been stressed due to frequent and unpredictable changes in light and temperature (only ambient room lighting and climate controls were used in this experiment). It would be better to control these factors to reduce external variation and remove a potentially stressful situation.

On the other hand, mortality may have been due to cannibalism rather than poor conditions. Exposure chambers were inspected weekly for mortalities, but only four individuals were found dead over the course of the experiment. If cannibalism does occur, regardless of whether isopods are consumed alive or already dead, this could lead to higher than expected Hg accumulation within isopod tissues.

### *Fluctuating Asymmetry*

Increasing the duration of contaminant exposure during rapid-growth may increase the sensitivity of FA tests. This might be achieved by beginning exposure when isopods are still in the manca (larval) stage, rather than when they are young adults (chapter 6). However, this would require using a method other than a Berlese funnel to separate individuals from the colony, as mancas in this study did not seem to respond to the light or heat of the Berlese funnel in any way. They appeared to be as likely to move towards the light source as away from it. It may be possible to obtain mancas by hand-selecting gravid females from the culture, but this would be a more time consuming process and would probably require a larger culture in order to obtain a reasonable sample size.

### *Behavior*

I recommend the use of behavioral endpoints that are more ecologically-relevant than those used in this study, as they would provide a specific stimulus, specific optimal response, and perhaps a more direct link to population or community dynamics. Examples are shelter use, predator avoidance, turning frequency, or phototaxis.

Numerous individuals climbed up the side of the Petri dish arena in this study and fell off, which caused those trials to be discarded from analysis. An arena that is even slipperier and more difficult to climb than a plastic Petri dish would improve future studies. Alternately, a chemical repellent might be used on the sides of the container, but only if a repellent exists that is localized enough to repel individuals that actually try to climb the side but not those on the arena floor that simply wander near the arena's edge.

For example, gardeners use copper to repel snails and slugs in this manner, as these animals will move up to the edge of a sheet of copper but will avoid direct contact with it.

I do not know of a repellent that works similarly in isopods, however.

Small sample size was a concern in the behavioral trials of this study. Sample size was limited by time and personnel availability. I scheduled only one day to both separate isopods from the exposure chambers and carry out behavioral trials. One way to improve time efficiency would be to design the experiment so that individuals were removed from the exposure chambers a day before behavioral trials, rather than on the same day. Another would be to hold behavioral trials on multiple days. This could be compensated for by using day as a block during statistical analyses of behavioral patterns.